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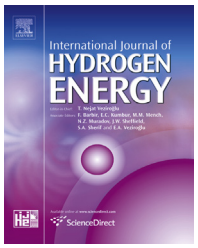

Influence of distiller's grains and condensed tannins in the diet of feedlot cattle on biohydrogen production from cattle manure

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ABSTRACT

Biohydrogen production from the manure of cattle fed diets containing corn dried distiller's grains with solubles (DDGS) diets was assessed. Four types of manure were obtained from cattle fed four diets (DDG, %of dietary dry matter): 0 (CK), 20 (DG20), 40 (DG40) and 40 plus 2.5% of dietary dry matter as condensed tannins (DG40CT) and evaluated for biohydrogen production using dark fermentation. Each treatment was evaluated in quadruplicate using 2 L continuously stirred biodigesters operating at 55 °C in batch culture with an organic loading rate of 20 g L^{-1} volatile solids and a total operation time of 4 d. Gas samples were taken daily to determine hydrogen production, and slurry samples were analyzed daily for volatile fatty acid concentration, total ammonia nitrogen, volatile solids degradation, and soluble ion concentration. The DG0 and DG40 treatments demonstrated the greatest hydrogen production, with DG40CT producing the least ($P < 0.001$). The inclusion of tannins in the diet of cattle had a negative effect on biohydrogen production from cattle manure, and thus the economic feasibility of using manure as a substrate in anaerobic digestion could be negatively impacted by the inclusion of tannins in the diet.

1. Introduction

Dried distiller's grains with solubles (DDGS) are an economically important co-product of ethanol production from corn and wheat [1]. Expansion of the bioethanol industry is ongoing, yielding 53 billion liters from the USA alone in 2011 [2]. In 2010/2011, approximately 40% of the corn harvest, or 128 Mt, was used for ethanol production [3]. Conversion of 1000 kg of corn into ethanol results in approximately 309 kg of DDGS (90% dry matter [DM]), resulting in production of ~43 Mt DDGS annually [2]. Because DDGS contain 20–35% crude protein and energy values on par or greater than corn, they have become an important component of beef cattle feeds in North America. In fact, 38% of distiller's grains produced in the USA that are not exported are consumed by beef cattle [4]. The effects of feeding DDGS on growth performance of feedlot cattle have been studied

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previously [5–7]and inclusion of DDGS in the diet dramatically increased N and P excretion, presenting a significant challenge with respect to manure nutrient management [6,8,9].

Condensed tannins (CT), also known as proanthocyanidins, are polyphenolic secondary plant metabolites that have been studied as feed additives for beef cattle [10,11]. Feed proteins and CT form complexes in a pH dependent manner, reducing protein degradation in the rumen while allowing for greater protein adsorption in the intestinal tract [11]. Advantages of including CT in the diet include: increased protein deposition, reduced urinary N excretion, parasite control, and bloat safety [11]. However, CT are not adsorbed in the digestive tract and are excreted in modified forms in feces, where they may alter N availability in the feces and potentially alter the soil microbial community when manure is land-applied [12,13].

Renewable energy production and environmental sustainability are currently major issues being addressed through the development of technologies such as ethanol production. Another potential source of renewable energy is biohydrogen, which can be produced from organic wastes via mixed microbial fermentation. Capture of hydrogen and other gases produced during anaerobic digestion can reduce greenhouse gas emissions from manure and reduce the discharge of nutrients into the environment [14]. During anaerobic digestion, carbohydrates, fats and proteins are fermented into intermediates such as volatile fatty acids (VFA), alcohols and ammonia. Hydrogen is produced by the hydrogenase enzyme when VFA and alcohols are converted into acetate as a means to re-oxidise the electron carriers NAD^+ and ferredoxin [15]. Several factors influence the fermentative production of hydrogen, including microbial ecology, temperature, pH, partial pressure of hydrogen, as well as the concentration and characteristics of the substrate [16].

Fermentative hydrogen production is a renewable energy source that has the potential to be used synergistically in the lifecycle of DDGS, providing an environmentally sound method for manure management (Fig. 1). The production of hydrogen via fermentation of organic wastes such as cattle manure has been increasingly studied in recent years and could be used to dispose of manure derived from feedlot cattle [17–19]. Biohydrogen production using DDGS as a substrate has also recently been investigated [20]. However, the influence of feed ingredients such as DDGS and CT on biohydrogen production from manures has not been assessed. The objectives of this study were to: 1) compare biohydrogen production from manure arising from feedlot cattle fed DDGS versus manure from cattle fed a barley grain-based diet; 2) determine the effect of CT in the diet on biohydrogen production from manure; and 3) to assess a molecular technique for predicting biohydrogen yield from manure prior to fermentation.

2. Materials and methods

2.1. Materials

Cattle manure was obtained from a total of 8 pens housing crossbred steers involved in a DDGS feeding trial as described

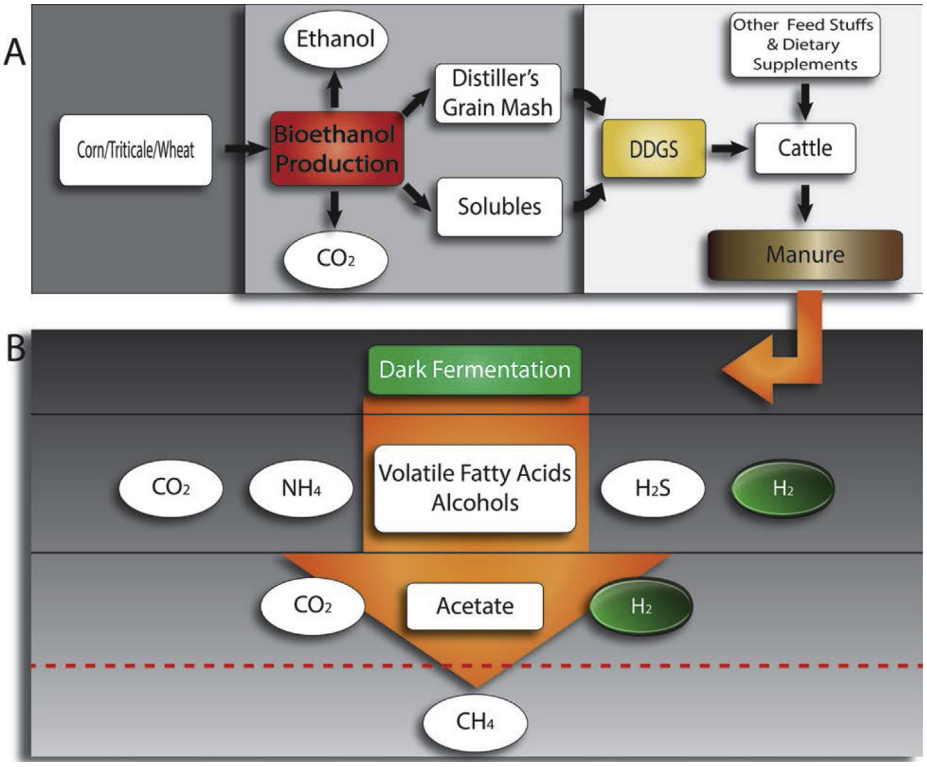

Fig. 1 – Schematic of bioethanol production from grains, resulting in dried distiller's grains with solubles (DDGS) which are fed to feedlot cattle (A). Manure produced from cattle is used as a substrate for biohydrogen production via anaerobic dark fermentation, which is a multi-step process involving substrate hydrolysis, acidogenesis, and acetogenesis (B). Under normal anaerobic conditions the final product is CH4, which is undesirable for biohydrogen production (dashed red line).

Table 1 – Dietary composition of cattle feed for each treatment prior to manure collection.

Ingredient	Cattle diet composition (%dry matter)			
	DG0	DG20	DG40	DG40CT
Barley silage	9	9	9	9
Barley grain, temper-rolled	86	66	46	46
Corn DDGS	0	20	40	40
Supplement[a,b]	5	5	5	2.5
Condensed tannins[c]	0	0	0	2.5

a Supplement contained 68% barley grain, 25% calcium carbonate, 3% NaCl, 2.5% dried molasses, 1.47% vitamin-mineral premix.
b Vitamins, minerals, and antibiotics per kg of diet: 50 mg Zn, 13 mg Cu, 24 mg Mn, 0.6 mg I, 0.25 mg Se, 0.2 mg Co, 8600 IU vitamin A, 430 IU vitamin D, 12 IU vitamin E, 33 mg monensin.
c Condensed tannins derived from *Acacia mearnsii*.

previously [21]. Details of the dietary composition of cattle on each of the 4 experimental diets can be found in Table 1. Fresh manure (5 kg, <24 h post-excretion) was collected from the cement pad within cattle pens with care to exclude soil and bedding materials. For each dietary group, 2.5 kg of manure was collected from duplicate pens (10 animals each), pooled, then homogenized by hand until uniform. Triplicate subsamples (10 g) were analyzed for volatile solids (VS) according to standard methods [22], while the remaining manure was stored at 4 °C until further use (<72 h). The experiment was performed in quadruplicate, with fresh manure obtained prior to each new experimental replicate.

Manure from each dietary treatment was prepared for biohydrogen production by heat-treatment in an oven at 100 °C for 1 h to inactivate methanogenic microbes and enrich for spore-forming hydrogenic bacteria [23]. After heat treatment, manure was mixed with distilled water and homogenized into a slurry (20 g L^{-1} VS) using a blender (Waring, Torrington, USA). For each treatment, 1.5 L of slurry was added to a 2 L bench scale batch fermenter (Minifors, Infors AG, Basel, Switzerland). Anaerobic conditions were ensured by flushing the fermenter headspace with helium gas. Fermenters were operated at 55 °C and continuously agitated via an impeller at 600 rpm for a total of 4 days.

2.2. Analyses

Biogas production was measured daily using a water displacement apparatus. Gas samples were taken daily from the fermenter head space using a 10 mL syringe, then stored in evacuated 6.8 mL Exetainers® (Labco Limited, Lampeter, UK) until analysis by gas chromatography (GC). The concentrations of H_2, N_2, CO_2, and CH_4 were determined using a 2-channel GC (Varian 4900 micro GC, Varian Inc., Palo Alto, USA) equipped with a thermal conductivity detector and a 2 m × 2 mm (inside diameter) stainless steel column packed with Porapak N (80–100 mesh) (Supelco, St. Louis, USA). Injector and column temperatures were kept at 110 °C and 40 °C, respectively for H_2, and N_2 analysis on Channel A, and 110 °C and 60 °C, respectively, for CH_4 and CO_2 analysis on Channel B. Channel A used argon as a carrier gas while helium was the carrier gas for Channel B, with the carrier gasses for both channels maintained at a static pressure of 150 kPa.

Slurry aliquots were taken daily immediately after gas sampling and frozen at −20 °C until further analysis. Aliquots were analyzed for VS according to standard methods [22]. Samples (2 mL) for VFA analysis were centrifuged at 13,000 g for 10 min, followed by acidification of the supernatant (1000 μL) with 25% m-PO_4 (200 μL). Samples were allowed to stand for 30 min and then centrifuged again at 13,000 g for 10 min. A 1000 μL sample of the supernatant was used for VFA determination using crotonic acid as an internal standard. VFA (acetate, propionate, butyrate, isobutyrate, valerate, isovalerate, caproate) were analyzed using a Varian 3800 GC (Varian Inc., Palo Alto, USA) equipped with a flame ionization detector at 250 °C and a fused-silica capillary column (25 m × 0.32 mm WCOT FFAP-CB). The oven temperature program was 80 °C for 5 min, ramp 20 °C min^{-1}–225 °C for 8 min. The injector was held at 280 °C and helium was used as the carrier gas with a flow rate of 5 mL min^{-1}. Total VFA concentration reported was the sum of the concentrations of those VFA analyzed. Slurry samples for NH_4^+ analysis were first centrifuged (10 min at 13,000 g), and the supernatant was diluted either 50 or 100 times in deionized water to a final volume of 5 mL. Concentration of NH_4^+ was determined colorimetrically using an Auto Analyzer 3 (Bran + Luebbe, Norderstedt, Germany) at 650 nm. Soluble ion concentrations (Ca, Cl, Mg, NO_2, NO_3, PO_4, K, Na, and SO_4) were determined in slurry supernatants after centrifugation and filtration through a 0.42 μm syringe filter via ion chromatography (ICS-1000 and DX-600, Dionex, Sunnyvale, USA).

Calorimetric determination was performed on 1 g dried and ground (<1 mm) solids from slurry samples using an Adiabatic 1241 oxygen bomb calorimeter (Parr, Moline, USA). Heats of combustion were determined by comparing the heat obtained from the sample to standardized material (benzoic acid = 26,460 J g^{-1}; Fluka, St. Louis, USA). Samples were combusted in a high-pressure oxygen atmosphere within a pressurized (30 atm) metal vessel. Energy released during the combustion was absorbed within the calorimeter and the resulting temperature change was recorded.

Triplicate 2 mL slurry subsamples were frozen and lyophilized for 4 d in a FreeZone Freeze Dry System (Labconco Corp., Kansas City, USA) followed by pooling of solids which were then finely ground using a Ball Mill (MM200; Retsch GmbH, Haan, Germany). The DNA was extracted from 100 mg of pooled solid sample using QIAamp DNA stool mini kit (Qiagen, Mississauga, Canada) according to the manufacturer's protocol and quantified using a Nanodrop 1000 Spectrophotometer (Thermo Scientific, Waltham, USA), then stored at −20 °C prior to real-time PCR analysis. Plant universal chloroplast *rbcL* gene (encoding ribulose bisphosphate carboxylase/oxygenase, Rubisco; Genbank accession no. X04975.1) was selected as a target sequence for quantifying DNA of plant origin. A primer set framing a 138-bp plant Rubisco gene fragment (Rub138; F: 5′-CTT GGC AGC ATT CCG AGT A- 3′; R: 5′-CCT TTG TAA CGA TCA AGA CTG G- 3′) was utilized [24]. Each PCR mixture contained (final concentrations) 1X iQ SYBR Green Master Mix (BioRad, Hercules, USA), 1 μg μL^{-1} BSA (New England Biolabs, Ipswich, USA), 0.2 μM each primer, and 1 μL of diluted sample DNA (≈10–20 ng) or standard DNA in a final volume of 25 μL.

Real-time PCR amplification was performed on a Mastercycler® ep realplex^4 (Eppendorf, Hamburg, Germany), using PCR conditions of 95 °C for 4 min; 45 cycles of 95 °C for 15 s, 60 °C for 1 min, and 72 °C for 15 s; and final extension at 72 °C for 5 min. Data were collected at the end of each elongation step and data analysis was performed using the Realplex software v.1.0 (Eppendorf, Hamburg, Germany). Real-time PCR quantification results were presented as $\log_{10}$ copy number g^{-1} dry weight.

Statistical analyses were performed using the general linear models module of StatSoft Statistica version 11.0.

3. Results and discussion

3.1. Hydrogen concentration and production

Repeated measures of hydrogen concentration over the duration of the experiment did not ($P > 0.05$) vary between treatments (data not shown). However, the maximum hydrogen concentration achieved in each treatment differed ($P < 0.001$) (Fig. 2c). Additionally, the time at which maximum hydrogen concentration was achieved differed by treatment, with DG0 reaching maximum concentration (49% of headspace) on average at 2 d, DG20 (45%) at 1 d, DG40 (47%) at 3 d, and DG40CT (37%) at 4 d. These results suggest an increased lag time associated with the 40% DDGS treatments, and that inclusion of CT further increased this lag. Cumulative hydrogen production (Fig. 2a) also reflected an increased lag time for DG40 compared to DG0 and DG20, although the variability of production was greatest for DG40. There was no discernible lag for DG40CT, mainly because hydrogen production was consistent over time, but at a lower rate than observed for the other treatments. Total hydrogen production (mL H_2 g^{-1} VS fed) varied according to treatment ($P < 0.001$) (Fig. 2b) with greatest production observed for DG0 and DG40, and least production for DG40CT.

3.2. Volatile fatty acid production

Production of VFA followed a similar pattern to hydrogen production according to treatment (Fig. 3a). Repeated measures of VFA concentration over the entire course of the experiment did not reveal differences among treatments ($P > 0.05$), but differences were observed at d 1–3 ($P < 0.05$), which also coincides with peak hydrogen production (Fig. 2a). The predominant VFA present in all treatments were acetic and butyric acids (data not shown), which is consistent with mixed acid fermentation carried out by clostridia (eq. (1) and (2)) [16].

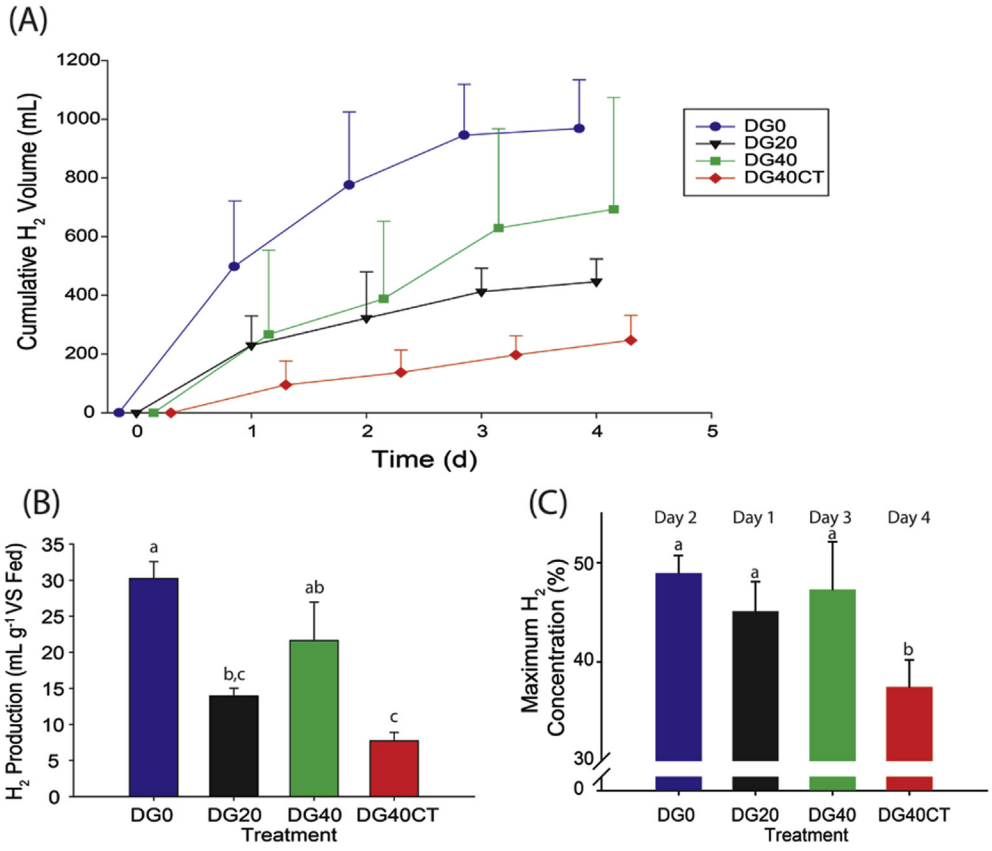

Fig. 2 – Comparison of cumulative hydrogen production during 4 d of fermentation between treatments (A), total hydrogen production expressed in relation to volatile solids (VS) fed (B), and the maximum hydrogen concentration observed along with the day of observation (C). Plots and bars represent averages ($n = 4$) and error bars SD. Significance was determined by ANOVA followed by Tukey's HSD tests and is represented by lower case letters above treatment groups (B,C).

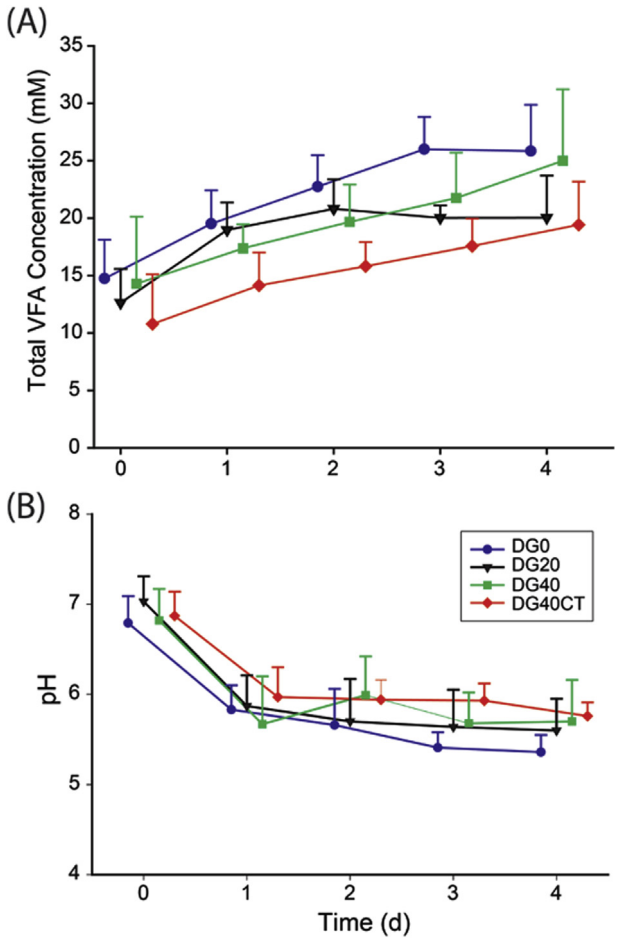

Fig. 3 – Concentration of volatile fatty acids (sum of acetate, propionate, butyrate, isobutyrate, valerate, isovalerate, caproate) produced over time during fermentation (A), and change in pH over time during fermentation (B). Plots represent averages ($n = 4$) and error bars SD.

$$\text{Glucose} + 2H_2O \rightarrow 2\,\text{Acetate} + 2CO_2 + 4H_2 \quad \Delta G^\circ = -182.4\ \text{kJ} \quad (1)$$

$$\text{Glucose} \rightarrow \text{Butyrate} + 2CO_2 + 2H_2 \quad \Delta G^\circ = -257.1\ \text{kJ} \quad (2)$$

Over time the proportion of acetic acid decreased while butyric acid increased in all treatments. However, for DG0 and DG40, the butyric acid levels rose more ($P < 0.05$) than in DG20 and DG40CT. This can be attributed to the higher hydrogen production in DG0 and DG40. As hydrogen accumulates due to the absence of hydrogenotrophic activity in the system, the more energetically favorable butyrate fermentation pathway is utilized, resulting in mixed acid fermentation (eq. (3)) [16].

$$4\,\text{Glucose} \rightarrow 2\,\text{Acetate} + 3\,\text{Butyrate} + 8CO_2 + 8H_2 \quad (3)$$

3.3. *Total ammonia nitrogen concentration*

Initial total ammonia nitrogen (TAN) concentration did not differ among treatments ($P > 0.05$), which is inconsistent with previous findings [8] that showed manure from cattle on the DG40 diet had significantly more TAN than other treatments. However, caution must be exercised when comparing these two studies because the previous study measured TAN from solid manure samples that had been stored for 14 days after the end of the feeding trial. In comparison, the TAN measurements taken here used manure from an earlier point in the feeding trial, the manure was fresh and not stored, and the samples were from slurry instead of solid manure. Repeated measures over time revealed differences ($P < 0.01$) in TAN accumulation between treatments, with the largest increase in concentration observed for DG40CT (Fig. 4). The fermentation of corn into ethanol removes starch from the grain, increasing the relative protein concentration of DDGS and in turn of manure from animals fed on DDGS diets [9,25]. Fermentation of protein results in the release of soluble NH_4^+ and explains the elevated TAN concentrations found in treatments containing DDGS. Condensed tannins are known to form stable complexes with proteins in a pH-dependent manner, reducing their availability for fermentation at pH near neutrality [11,26]. However, tannin-protein complexes become less stable as pH decreases, releasing protein during fermentation and making it available for conversion to NH_4^+ as observed for DG40CT.

3.4. *Volatile solids degradation*

Degradation of VS differed according to treatment ($P < 0.05$), as DG40CT displayed the least amount of VS loss (Fig. 5). For DG0, DG20, and DG40, >40% of VS were degraded in during the 4 day period, while 34% of VS were degraded in DG40CT. The lower VS degradation observed for DG40CT was likely related to the binding of protein substrate by tannins. As pH decreased during fermentation, protein-tannin complexes dissociated to make protein available for fermentation (Fig. 3b). However, CT have demonstrated antimicrobial

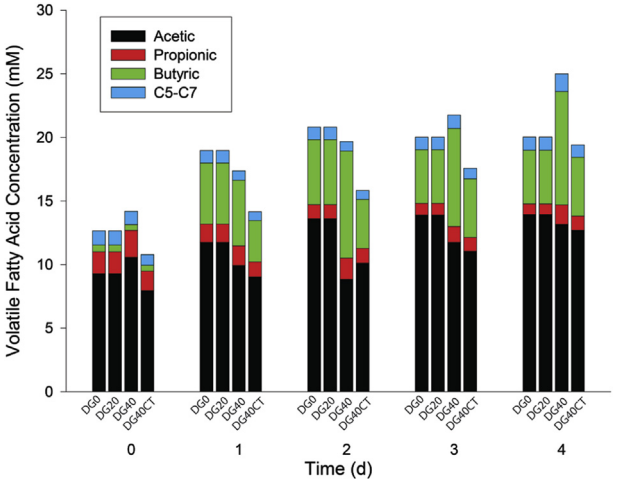

Fig. 4 – Composition of total volatile fatty acid (VFA) profile by treatment, grouped by time. Cumulative height of each bar represents total VFA, while each colored segment represents the contribution of particular VFA to the total. Segments labeled C5–C7 correspond to the sum of VFA with carbon chain length ranging from 5 to 7.

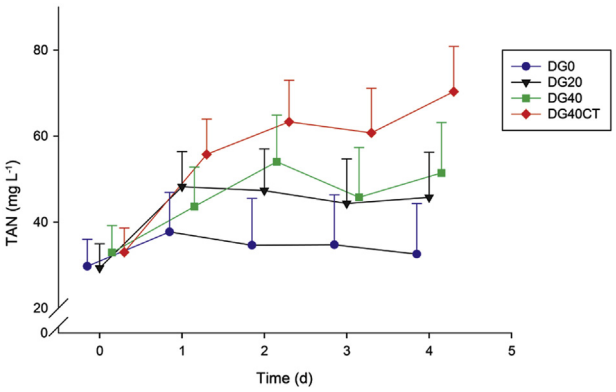

Fig. 5 – Changes in concentration of total ammonia nitrogen (TAN) over time during fermentation. Plots represent averages ($n = 4$) and error bars SD.

properties [27], possibly inhibiting the microbial community responsible for hydrogen production in this study. Tannins added to *in vitro* ruminal fermentation studies of wheat and corn have been reported previously to have inhibitive effects [28]. In addition to their antimicrobial properties, CT have been demonstrated to reduce the digestibility of starch [29], and form complexes with other carbohydrates and minerals [30]. While adaptation of ruminant microbial populations to CT has been demonstrated, in this study the microbial community was not given time to adapt before starting the experiment. The antimicrobial effect of tannins is poorly understood and varies widely depending on the tannin source, bacterial species, and acclimation time [31]. It is possible that hydrogen yield from DG40CT could be improved through continuous culturing in the presence of CT if adaptation by the hydrogenic bacterial community occurs.

In this study the hydrogenic community was selected by heat-shocking manure prior to fermentation. Because of this, the microbial community present in each inoculum may have varied based on differences in dietary treatment. The differences observed in hydrogen production and VS degradation may therefore be due in part to microbial community composition as well as treatment-specific substrate variation.

3.5. Soluble ion concentrations

Concentrations of soluble Ca^{2+} increased over time for all treatments (Table 2), likely due to the decrease in pH resulting from VFA production which leads to enhanced Ca^{2+} solubility (Fig. 3a and b). No treatment effect was observed in relation to changes in Ca^{2+} ($P > 0.05$). The amount of P released during biodigestion, as indicated by the difference in final compared to initial concentrations, tended to decrease with inclusion of DDGS and was lower ($P < 0.05$) in DG40CT than DGO. This was expected given the same trend was observed for VS degradation. In anaerobic environments, SO_4^{2-} can be reduced to H_2S by the activity of sulfate reducing bacteria. This phenomenon explains the decrease in SO_4^{2-} concentration over time for all treatments (Table 2). However, differences ($P < 0.05$) were observed between treatments for sulfate reduction, with the level of sulfate reduction observed for DG40CT lower compared with DG0 and DG20. This finding suggests that the presence of tannins may suppress the activity of sulfate-reducing bacteria or the formation of SO_4^{2-} from S containing amino acids. Further analysis is needed to verify the repeatability of this observation. Suppression of SO_4^{2-} is desirable because the production of H_2S scavenges hydrogen, lowering biohydrogen yields. In this study CT had broad inhibitive effects on biodegradation and biohydrogen production, but at lower concentrations CT could potentially be beneficial if the activity of sulfate-reducing bacteria can be inhibited without compromising biohydrogen production.

3.6. Total energy content and plant DNA quantification

To determine whether the results observed for hydrogen production were due to differences in the total gross energy content of the initial manure, bomb calorimetry was performed. Results from a companion metabolism study with cattle indicated that the apparent total tract digestibility of organic matter was: DG0 (82%) > DG20 (78%) > DG40 (75%) > DG40CT (73%) (unpublished data). Differences in digestibility suggest that the gross total energy content (cal g^{-1} DM) of the manure differed amongst dietary treatments, confirming our observations ($P < 0.001$) (Fig. 6). Similar to the results of the metabolism study, the greatest gross energy content was observed for DG40 and DG40CT, while the lowest

Table 2 – Soluble ion concentrations[a] in effluent before and after fermentation.

Treatment	Ca	Cl	Mg	NO_2	NO_3	P	K	Na	SO_4
Day 0									
DG0	101.3 ± 17.7	50.8 ± 6.9	5.5 ± 11.9	0.1 ± 0.1	0.3 ± 0.1	60.1 ± 14.7	83.2 ± 7.8	36.1 ± 11.8	11.7 ± 1.5
DG20	72.1 ± 12.3	55.9 ± 13.5	77.7 ± 12.4	0.1 ± 0.0	0.2 ± 0.0	62.4 ± 24.1	105.2 ± 41.6	22.4 ± 9.3	9.9 ± 2.9
DG40	82.5 ± 20.2	56.0 ± 7.4	114.3 ± 25.2	0.1 ± 0.0	0.2 ± 0.0	106.1 ± 78.4	96.8 ± 34.9	22.2 ± 14.4	11.2 ± 1.3
DG40CT	69.6 ± 17.4	64.3 ± 20.6	71.6 ± 10.6	0.1 ± 0.0	0.2 ± 0.0	98.2 ± 37.1	148.3 ± 33.7	41.7 ± 28.6	8.8 ± 2.9
Day 4									
DG0	280.8 ± 76.0	42.1 ± 4.9	89.3 ± 20.6	0.1 ± 0.1	0.2 ± 0.1	273.8 ± 111.0	79.5 ± 3.0	42.8 ± 12.1	4.6 ± 4.0
DG20	248.4 ± 35.4	52.3 ± 14.2	114.3 ± 14.3	0.1 ± 0.1	0.2 ± 0.1	290.4 ± 89.7	110.5 ± 48.7	29.4 ± 13.2	2.8 ± 4.3
DG40	193.7 ± 87.8	53.4 ± 6.6	149.4 ± 24.2	0.0 ± 0.0	0.2 ± 0.0	286.9 ± 85.4	100.4 ± 33.7	44.4 ± 11.4	7.5 ± 6.4
DG40CT	148.4 ± 24.3	63.0 ± 17.5	98.5 ± 11.4	0.1 ± 0.1	0.2 ± 0.1	235.8 ± 60.5	153.9 ± 19.7	67.7 ± 42.0	7.9 ± 4.6

a All concentrations expressed as μg mL^{-1} ± SD.

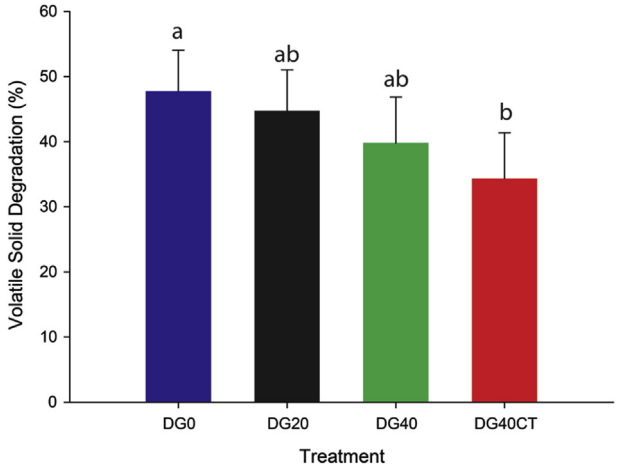

Fig. 6 – Percentage degradation of volatile solids (VS) between Day 0 and Day 4, according to treatment. Bars represent averages ($n = 4$) and error bars SD. Significance was determined by ANOVA followed by Tukey's HSD tests and is represented by lower case letters above treatment groups.

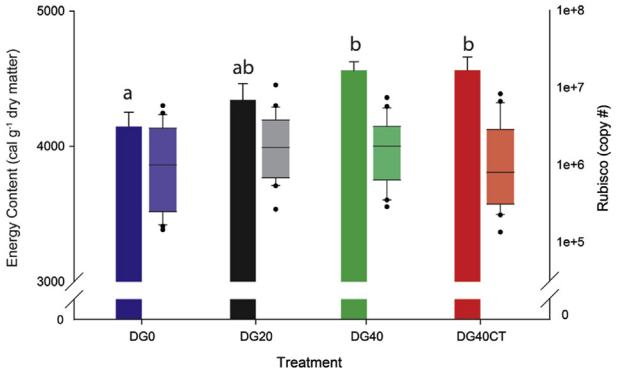

Fig. 7 – Gross energy content of initial manure samples as determined by bomb calorimetry (left axis) and copy number of the plant Rubisco gene fragment expressed on a logarithmic scale, as determined by quantitative PCR (right axis). For each treatment, the left bar indicates energy content and the right bar indicates Rubisco copy number. Significance was determined by ANOVA followed by Tukey's HSD tests and is represented by lower case letters above treatment groups.

was found in DG0. Differences in gross energy content of the manure samples did not correlate with the differences observed in hydrogen production. However, gross energy content as determined by calorimetry does not necessarily represent bioavailable energy, due to the presence of recalcitrant compounds such as lignin, hemicelluloses, or celluloses that would not have been fully degraded over the 4 d of fermentation used in this study. The inverse relationship between hydrogen production and gross energy content exhibited by DG0 and DG40CT suggests that manure from DDGS treatments contained less bioavailable energy. This result was expected, as starch is removed from corn that has been converted to bioethanol, leaving behind a higher concentration of protein and recalcitrant carbohydrates in the remaining DDGS [25]. With further degradation of the DDGS in the digestive system of cattle, it could be expected that manure based on DDGS would have a higher proportion of recalcitrant compounds remaining than DG0.

To further evaluate the differences in potential digestibility of manure substrate during biodigestion, a real-time PCR assay quantifying the presence of a multi-copy plant gene, Rubisco (ribulose-1,5-bisphosphate carboxylase oxygenase), was performed (Fig. 7). The presence of plant-specific genes has previously been directly correlated to the level of degradation of plant material in the rumen [32]. If the correlation between Rubisco copy number and biodegradability was also true for manure, the number of Rubisco gene copies found in DG40CT would be expected to be significantly lower than in DG0. This would indicate that plant material in DG40CT was degraded to a greater extent than in DG0 and would help explain the disparity in hydrogen production based on treatment (Fig. 2). Extending this concept further, quantification of Rubisco gene copies in manure could potentially be used as a predictive molecular tool to estimate the amount of bioenergy that could be produced from a particular manure source. However, our results indicated that there was no relationship between treatment and Rubisco copy number ($P > 0.05$). This finding means that although the gross energy content of the manures varied according to treatment, the disparity could not be explained by differences in the level of degradation of plant material after digestion in cattle according to qPCR. The original qPCR technique [32] showing a correlation between DNA and dry matter loss used fresh plant material as animal feed, but in this study plant cell walls in DDGS manures were disrupted during both the bioethanol fermentation process and animal digestion prior to biohydrogen production. These additional processing steps would have liberated much of the DNA from plant cells prior to biohydrogen production, breaking the correlation between DNA and degradability, and making the qPCR assay unsuitable as a predictive technique for the purposes tested here.

The best explanation for the disparity in biohydrogen production observed between treatments is therefore related to the limited digestibility of DDGS components remaining in manure after animal digestion and the inhibitive effects of CT on the biohydrogen production process. Intuitively this explanation makes sense, as the amount of gross energy available in corn is reduced twice, once during ethanol production and once during animal digestion, prior to biohydrogen production. The additional processing step limits the amount of energy remaining in DDGS manures that is available for biohydrogen production. A complete energy balance, factoring the initial energy potential of corn and the energy derived in the form of ethanol, animal weight gain, and biohydrogen production, needs to be performed to determine the strategy that maximizes energy recovery while prioritizing energy flow to the most desirable product. Increasing the flow of energy to one of the three potential outputs outlined here seems likely to reduce the energy available for downstream recovery without additional treatment steps. For example, if the efficiency of DDGS conversion to weight gain could be

increased further in cattle, it could be expected that biohydrogen yields from DDGS manure would be further reduced. However, with the addition of a hydrolysis step designed to make lignocellulosic components available for fermentation during biohydrogen production, additional energy recovery may be possible. It is important to recognize that alterations to one process can have far reaching consequences when long derived-value chains exist, such as in the case of corn destined for bioethanol production. Further research to quantify and predict the impact of fluxes in energy removal along the entire life cycle of corn and other biofuel feedstocks is necessary for creating a stable and predictable bio-economy.

4. Conclusions

Cattle diet affected biohydrogen production from manure using dark fermentation. Biohydrogen production potential from cattle manure was reduced when DDGS were included in the diet and CT were particularly inhibitive to fermentation. There was no correlation found between biohydrogen production and a qPCR assay targeting a Rubisco gene fragment, making the assay unsuitable for predicting biohydrogen yield. Manure is a major resource for renewable bioenergy production (biohydrogen and methane) throughout the world given its abundance, low cost, and the benefits of treatment before release to the environment. Given the increase in research related to ruminant dietary additives for improving feed efficiency, further evaluation of the impact of such amendments on the bioenergy potential of manures is necessary. In addition, life cycle assessments of greenhouse gas emissions from agriculture need to account for the net effects of modified feeding practices by including bioenergy as part of the total equation. Changing livestock feed sources cumulatively affects emissions from feed production, enteric release, and manure management.

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